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Evaluation of *spa*-typing of methicillin-resistant *Staphylococcus aureus* using high-resolution melting analysis

Running title: HRM *spa*-typing of MRSA

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Summary

Objective

spa-typing of methicillin-resistant *Staphylococcus aureus* (MRSA) has been widely used in clinical diagnostics and epidemiological studies. We aimed to evaluate high-resolution melting (HRM) as a rapid and cost effective method, to replace DNA-sequencing, for *spa*-typing in a global collection of 50 MRSA isolates.

Methods

The polymorphic X region of *spa* gene was amplified by colony PCR using SensiMix™ HRM kit and melting temperature (*Tm*) and melting curves of the amplicons was analyzed in close tubes using a Rotor-Gene 6000 instrument.

Results

Fifteen out of nineteen *spa*-types each had distinct *Tm*, which was sufficient to unambiguously type each of these *spa*-types. The remaining 4 *spa*-types cannot be separated by *Tm* alone: t008 and t2770 shared *Tm* (80.3^{o}C) and t021 and t311 shared *Tm* (80.0^{o}C). But, they can be separated based on the shapes of their melting curves. There are discrepancies between ours and previous studies, suggesting that standardization reminds a challenge for cross references.

Conclusion

HRM-s*pa*-typing is reproducible, simple, rapid and cost-effective. t037 is prevalent in Brazil and Sudan while diverse *spa*-types are found in Scotland and Saudi Arabia. Standardization is required for cross-references between labs globally.

Keywords: High-resolution melting (HRM), *spa*-types, Melting temperature (*Tm*), Methicillin resistant *Staphylococcus aureus* (MRSA).

Highlights

- DNA-sequencing and application of BioNumarics software have identified 19 *spa*-types in 50 isolates in a global MRSA collection. t037 is prevalent in Brazil and Sudan while diverse *spa*-types are found in Scotland and Saudi Arabia.
- DNA-sequencing based typing is time consuming and expensive, license for BioNumarics software alone costs ~ £6000 GBP for 3 years.
- Fifteen out of 19 *spa*-types are unambiguously identified by HRM. This procedure is rapid, 2 hour per run for 72 samples, and cost effective than DNA sequencing.
- *spa*-types with shared *Tm* can be distinguished by the shapes of the melting curve, which requires expertise training.
- There are discrepancies in *Tm* values for a few *spa*-types from 3 studies in 3 independent laboratories, which highlight the need for optimization and standardization for cross reference.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains to be a major cause of infections in community and healthcare settings that imposes significant threat to the public health (1, 2). Molecular typing methods are vital in rapidly identification of the prevalent strains which is important for active surveillance and controlling the spread of the disease. The polymorphic region of the gene encoding staphylococcal protein A (*spa)* has been found to be highly discriminatory that is useful in investigating both the local and global epidemiology of *S. aureus* (3-9). The complexity and high running cost of PCR-sequencing have limited to perform in developing countries (10). Recently, high-resolution melting (HRM) based *spa*-typing has been described as a rapid and cost effective method for genotyping locally predominant isolates (10, 11). To test the accuracy and reliability of HRM based *spa*-typing, we have analyzed a global collection of 50 MRSA isolates and demonstrated that HRM can accurately *spa*-type majority of these isolates.

Material and Methods

MRSA isolates

We randomly selected 50 clinical MRSA isolates from Scotland (n=22), Brazil (n=13), Sudan (n=3) and Saudi Arabia (n=12) between 2005 and 2012. All isolates were cultured and identified as *Staphylococcus aureus* as described previously (12). Methicillin resistance phenotype was confirmed according to the British Society for Antimicrobial Chemotherapy (BSAC) standards using Vitek2 system (Biomerieux, USA). An isolate was considered as methicillin resistance when the minimal inhibitory concentration (MIC) breakpoint of oxacilin is > 2 mg/L and cefoxitin > 4 mg/L (13).

DNA-sequencing for s*pa*-typing

The polymorphic regions of the *spa* gene were amplified and sequenced for all isolates, as previously described (5, 14). The sequence data were analyzed using *spa*-typing plugin in BioNumerics v.5.1 (Applied Maths).

HRM analysis for *spa*-typing

The polymorphic X region of *spa* gene was amplified in a Rotor-Gene 6000 instrument (Qiagen) by colony PCR using SensiMix™ HRM kit (Bioline) as previously described by Shopsin B. *et al* (3) . In brief, 20 µl PCR reaction was set up, containing 0.8 µl Eva-Green, 10 µl SensiMix™, 1 µl of each primer (100µM; 1095- forward 5`-AGACGATCCTTCGGTGAGC-3`and 1517 reverse 5`-GCTTTTGCAATGTCATTTACTG-3`) and 20 ng of the template DNA and programmed as following: a hold at 95°C for 10 min followed by 35 cycles of 95°C for 20s, 56°C for 20s and 72°C for 22s. The high-resolution melting of the amplicons was performed between 70–95°C with a stepwise increase of 0.05°C. The melting temperatures (*Tm*) were determined by the negative derivative of decreased fluorescence over increased temperature (*df/dt*), using the proprietary software (version 1.7.34). The sharpes of the melting curves are veiwed with the same software.

Results and Discussion

By use of DNA-sequencing and *spa*-typing plugin in BioNumerics, 19 *spa*-types were identified among the 50 isolates. The *spa*-type t037 was the major *spa*-type that was prevalent among Brazilian (12/13 isolates) and Sudanese isolates (3/3 isolates). Scottish and Saudi Arabian isolates were quite diverse; eight *spa*-types were observed among 22 isolates from Scotland and 9 *spa*-types among 12 isolates from Saudi Arabia (Table 1).

All the 50 isolates were then subjected to HRM analysis; 15 out of 19 *spa*-types each had distinct *Tm* which unambiguously assigned 44 isolates (Table 1, Fig. 1). The melting curve

within a *spa*-type was highly homogenous. However, despite the difference in the GC content between t008 and t2770 (43.7 mol% and 41.5 mol%, respectively), these *spa*-types shared the same *Tm* (80.3°C; Fig. 2A). Similarly, t021 and t311 could not be separated from each other; they shared a *Tm* of 80.70°C probably due to the fact that they have same 44.9 mol% of the GC content (Fig. 2B). These results are in agreement with Stephens *et al*. (11) where two *spa*-types could not be separated from each other, based on their *Tm*. It has been suggested that shapes of the melting curves are also important in determining the *spa*-types (11, 15, 16) and we have also noticed minor variations in the shapes of melting curves between t008 & t2770, and t021 & t311 (Fig. 2A and B). These variations in the shape of melting curves were reproducible but a bit complex for un-experienced users to confidently predict the correct *spa*-types which highlights the challenge in optimizing HRM based *spa*-typing for the growing number of *spa*-types of MRSA.

We also noticed some discrepancies in the *Tm* values between this study and previous investigations by Chen, *et al* and Stephens *et al*, (10, 11); (Table 2). The *Tm* values for t037 were 80.9, 83.6, 80.6; and for t002 were 81.6, 84.1, 81.2; according to Stephens *et a*l. (11), Chen *et al*. (10) and this study, respectively. Stephens *et al*. used Platinum SYBR-Green qPCR Super Mix-UDG (Life Technologies) on a Rotor Gene 6000 instrument (Qiagen) and we have used SensiMix™ HRM (Bioline) mix with Eva-Green dye on a Rotor Gene 6000 instrument (Qiagen) (11). Chen *et al*. used LightCycler 480 HRM Master Mix containing ResoLight dye on a LightCyclerNano real-time PCR system (Roche) (10). The *Tm* values both for t037 and t002 were relatively close between this study and Stephens *et al*., than Chen *et al*., suggesting the same instrument might provide similar *Tm* values for a *spa*-type and minor variations may have been caused by different reaction mixes that contained different reporting dyes. More discrepancies in the *Tm* values between Stephens *et al*. and Chen *et al*.

were observed for additional *spa*-types (Table 2). Therefore, different instruments and reagents (dye in the reaction mix) may result in discrepancies in the *Tm* values of a *spa*-type. Taken all together, we conclude that HRM- *Spa* typing is useful due to its reproducibility, simplicity, rapid and low cost. Standadization is needed for laboratory screening of *Staphylococcus aureus* spa-typing globally. For extension of its application to all *spa*-types and cross references among laboratories worldwide, it is necessary to standardize and optimize the experimental conditions in each of the laboratories.

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Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Figure legends:

Figure 1: Comparison of different *spa* polymorphic region X HRM curves obtained from MRSA isolates. (A) Negative derivative of florescence over temperature (*df/dt*) plots displaying distinguishable HRM profiles. (B) Normalization data curve depicts the decreasing fluorescence *vs* increasing temperature. (C) Difference graph demonstrating the accurate reproduction of 8 *spa* HRM profiles in a run experiment.

Figure 2: Melting curves shapes allowed assignments of *spa*-types share same *Tm*. (A) characteristics of melting curve shapes for t2770 and t008 respectively; they had identical *Tm* 80.3. (B) Characteristics of melting curve shapes of t021 and t311 respectively; they had identical *Tm* 81.0.

Table 2: Comparison of *Tm* obtained from three independent studies

Tm	HRM-*spa*-typing by		
	Stephen *et al*, 2008	Chen *et al*, 2013	Present study, 2015
79.4			t1544
79.5			t344
79.6	t123		
79.7	t352, T065 *		t044
79.8	t186		t131
80.0	t190		
80.1			
80.2			t304
80.3			t2770, t008 * *, ^
80.4	t437 $		t138
80.5			
80.6	t127, t008 *		**t037 #**
80.7	t019 $		t363
80.8			t11986
80.9	**t037 #**, t1155 *		t018 * *
81.0	t216		t311, t021 ^
81.1	t631		
81.2			**t002 #**
81.3	t018 * *		t4573
81.4			t020
81.5			
81.6	**t002 #**		
81.7			
81.8			t4291
81.9			t032 * * *
82.0			
82.1		t9469	
82.2	t202		
82.3		t1081	
82.5		t9377	
82.6		t4677	
82.9		t701	
83.1		t437 $	
83.2		t121	
83.3		t019 $	
83.6		**t037 #**	
84.0		t032 * * *	
84.1		**t002 #**	
84.3		t9970	

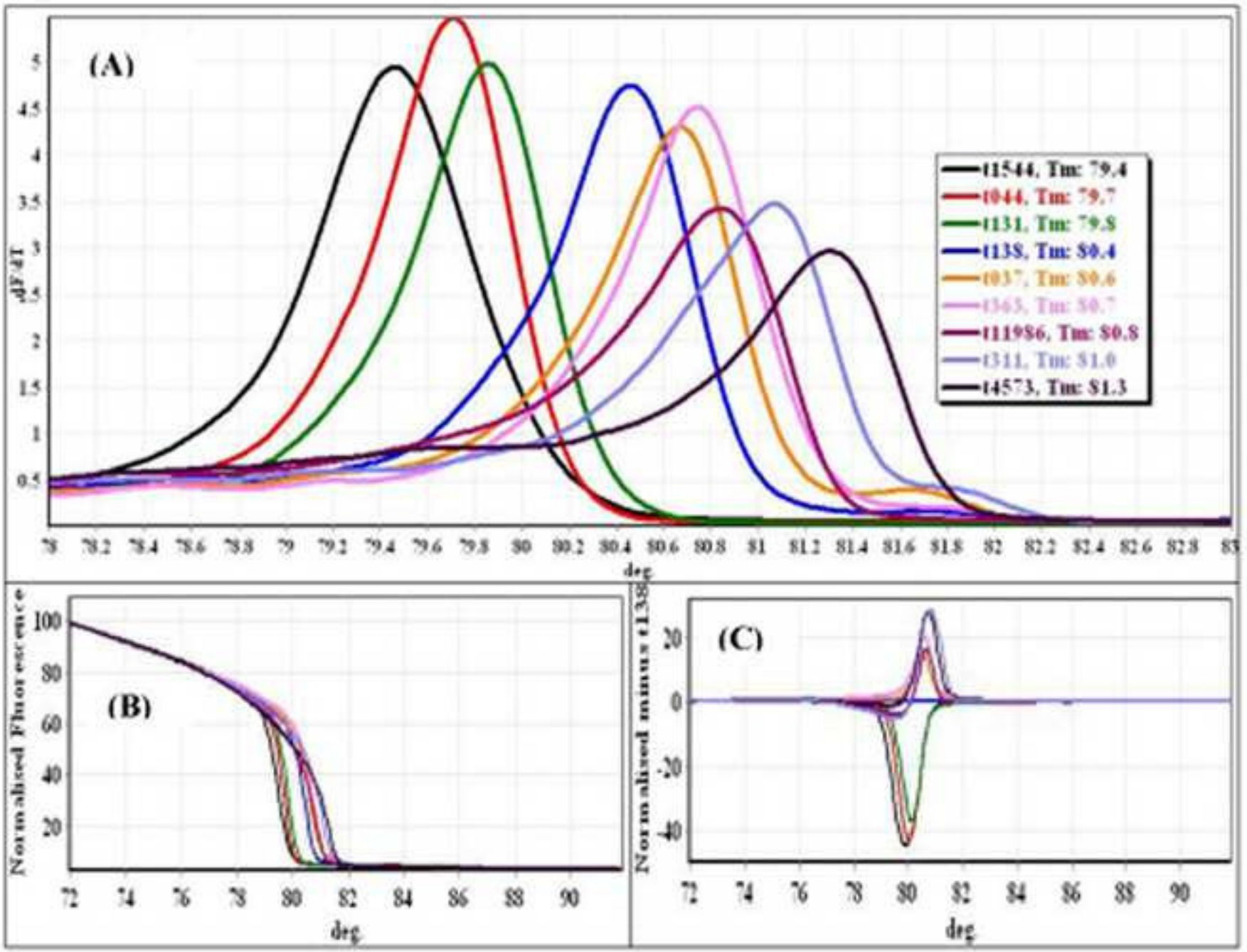
* Tm cannot distinguish *spa*-types in Stephen *et al* study.

** Tm discrepancy of *spa* types between our study and Stephen *et al*.

*** Tm discrepancy of *spa* type between our study and Chen *et al*.

$ *Tm* discrepancy of *spa* types between Stephen *et al* and Chen *et al* studies.
# *Tm* discrepancy in the three studies (text in **Bold**).
^ melting curve shape can distinguish *spa*-types in our study.

Figure


(A)
dF/dT
t1544, Tm: 79.4
t044, Tm: 79.7
t131, Tm: 79.8
t138, Tm: 80.4
t037, Tm: 80.6
t365, Tm: 80.7
t11986, Tm: 80.8
t311, Tm: 81.0
t4573, Tm: 81.3
deg
(B)
Normalised Fluorescence
deg
(C)
Normalised minus t138
deg

Figure

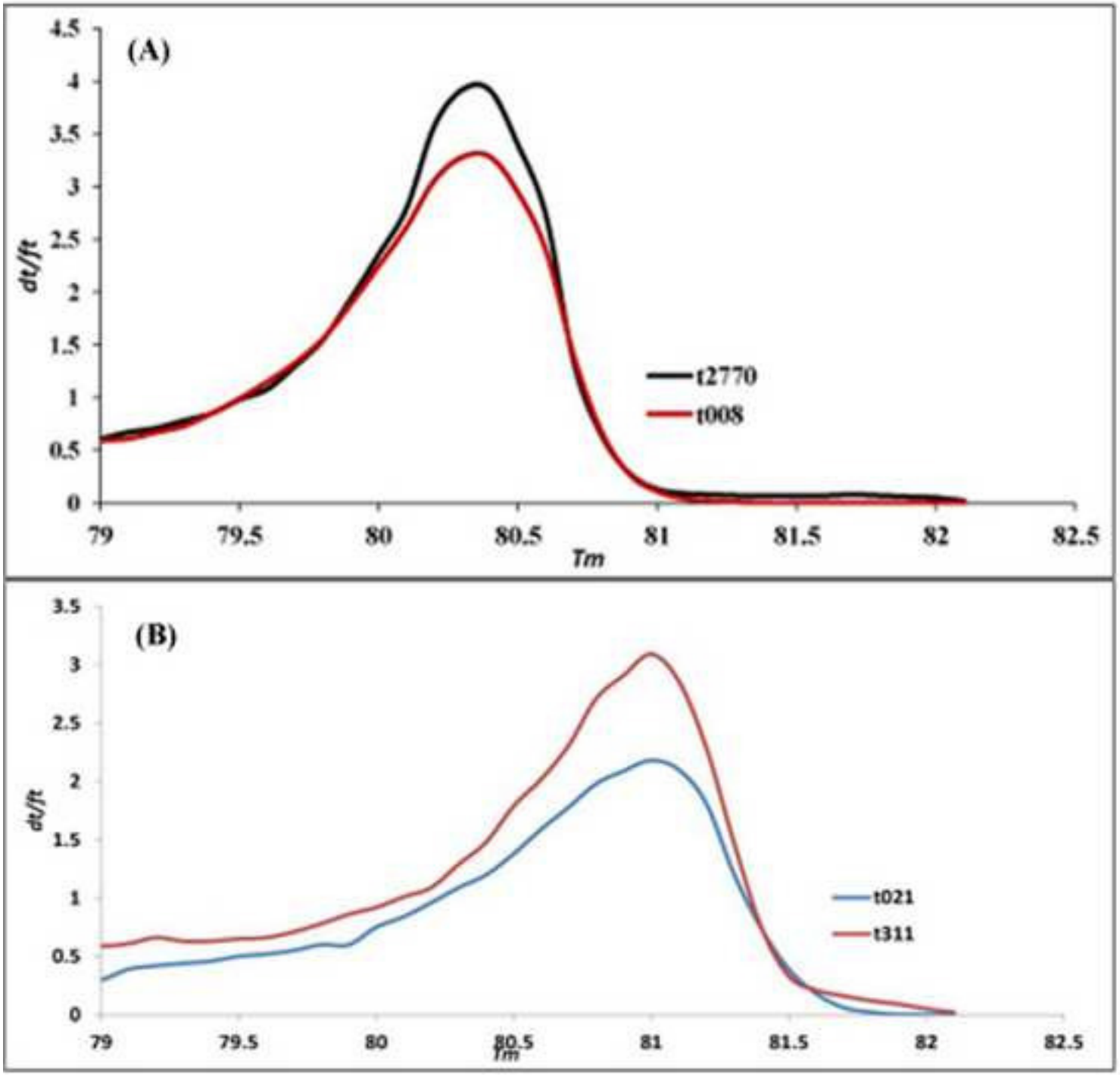


Table 1: HRM and *spa*-sequence types of the 50 MRSA isolates, and the frequencies in countries of origins.

HRM	*Tm*	*Spa*-type	Repeat of *spa* type	Size-bp	CG%	Country (N. of *spa* types/total)
1	79.4	t1544	07-22-34	72	44.4	Saudi Arabia (2/12)
2	79.5	t344	09-02-16-13-34	120	50	Scotland (5/22)
3	79.7	t044	07-23-12-34-34-33-34	168	41.7	Saudi Arabia (1/12)
4	79.8	t131	07-23-12-34-33-34	144	42.3	Saudi Arabia (1/12)
5	80.2	t304	11-10-21-17-34-24-34-22-25	216	43.5	Saudi Arabia (1/12)
6	**80.3***	**t2770**	**07-23-12-12-21-17-34-34-33-34**	**240**	**42.5**	**Saudi Arabia (2/12)**
		t008	**11-19-12-21-17-34-24-34-22-25**	**240**	**43.7**	**Scotland (1/22)**
7	80.4	t138	08-16-02-25-17-24	144	45.1	Brazil (1/13)
8	80.6	t037	15-12-16-02-25-17-24	168	45.23	Brazil (12/13) and Sudan (3/3)
9	80.7	t363	15-16-02-25-17-24	144	45.8	Saudi Arabia (1/12)
10	80.8	t11986	04-44-33-31-31-12-34-16-12-25-22-34	285	43.5	Saudi Arabia (1/12)
11	80.9	t018	15-12-16-02-16-02-25-17-24-24-24	264	44.86	Scotland (6/19)
12	**81.0***	**t311**	**26-23-17-34-20-17-12-17-16**	**216**	**44.9**	**Saudi Arabia (1/12)**
		t021	**15-12-16-02-16-02-25-17-24**	**216**	**44.9**	**Scotland (2/22)**
13	81.2	t002	26-23-17-34-17-20-17-12-17-16	240	45.4	Scotland (2/22)
14	81.3	t4573	07-23-13-23-31-05-17-25-16-28	240	45.0	Saudi Arabia (2/12)
15	81.4	t020	26-23-31-29-17-31-29-17-25-17-25-16-28	312	44.5	Scotland (1/22)